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D117N in *Cypher/ZASP* may not be a causative mutation for dilated cardiomyopathy and ventricular arrhythmias

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Dilated cardiomyopathy (DCM) and malignant ventricular arrhythmias are important causes of congestive heart failure, heart transplantation, and sudden cardiac death in young patients. *Cypher/ZASP* is a cytoskeletal protein localized in the sarcomeric Z-line that has a pivotal role in maintaining adult cardiac structure and function. The putative mutation p.(D117N) in *Cypher/ZASP* has been suggested to cause systolic dysfunction, dilated left ventricle with hypertrabeculated myocardium, and intraventricular conduction disturbance, based on two reported sporadic cases. In two unrelated Bedouin families, one with pediatric DCM and the other with DCM and ventricular arrhythmias at young adulthood searching for the causative mutation by exome sequencing we identified the p.(D117N) variant in *Cypher/ZASP*. However, p.(D117N) did not segregate as the causative mutation in these families, i.e. it was not present in some patients and was found in several individuals who had no clinical manifestations. Furthermore, the carrier frequency in the Bedouin population of origin is estimated to be 5.2%, which is much higher than the incidence of idiopathic DCM in this population. Thus, our data support the notion that the p.(D117N) variant in *Cypher/ZASP* is not a causative mutation in the families tested by us. The results also indicate that at least in some cases, the p.(D117N) in *Cypher/ZASP* is not a causative mutation and the role of D117N in *Cypher/ZASP* in cardiac pathologies should be further clarified and re-evaluated.

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INTRODUCTION

Dilated cardiomyopathy (DCM) is genetically transmitted in 30–40% of cases. Genetic heterogeneity has been identified with mutations in multiple cytoskeletal and sarcomeric genes causing the phenotype. Isolated non-compaction of the left ventricular myocardium (INLVM) with a hypertrophic dilated left ventricle, ventricular dysfunction, and deep trabeculations, is also inherited, and some of the genes identified to date differ from those causing DCM. The mechanistic links between genetic cardiomyopathies and arrhythmias are not fully defined. While cardiac dysfunction by itself clearly increases the risk of malignant arrhythmias, some patients die suddenly before symptomatic heart failure develops. In others, the arrhythmic phenotype can occur without any sign of mechanical dysfunction.¹ Mutations in the Z-band alternatively spliced PDZ motif (*ZASP1/ Cypher/LDB3*) gene that encodes a Z-disc protein have been identified in patients with DCM, INLVM, and various cardiac arrhythmias.^{2,3} *ZASP1* is one of the major components of the Z-Disc proteins in cardiac muscle,⁴ which has an important role in stabilizing the Z-Disc structure through its PDZ-mediated interaction with α -actinin-2, the main component of the Z-Disc actin cross-linker, and F-actin, the main cyto-architectural protein of cardiomyocytes⁵ as well as others.⁶ Global loss of normal *ZASP1* expression or ablation of the murine *ZASP* homolog *cypher* can disorganize both sarcomere and cytoskeleton, leading to severe

cardiomyopathy and skeletal myopathy in mice and humans, termed *zaspopathy*.⁷ Cardiac-specific ablation of the murine *cypher* led to dilated cardiomyopathy and death before 23 weeks of age.⁸

A putative mutation p.(D117N) in *ZASP1* was reported in two white individuals who suffered from systolic dysfunction, dilated left ventricle with hypertrabeculated myocardium, and intraventricular conduction disturbance. Both patients showed sporadic heterozygote mutation. One of the patients had a family history of sudden cardiac death.² The *ZASP1*-D117N also served to suggest a mechanism by which a mutation that disrupts a structural protein can cause electrophysiological remodeling that may contribute to conduction abnormalities. In this context, the effects of *ZASP1*-D117N on the sodium channel *Nav1.5* were studied by patch-clamp recordings of human embryonic kidney-293 cells and neonatal rat cardiomyocytes by Xi and colleagues.⁹ Their results demonstrated that *ZASP1*-D117N attenuated *I_{Na}* by 27 and 32%, respectively, and right shifted the activation and inactivation curves compared with *ZASP1*-wild-type. Based on computational modeling, these effects of *ZASP1*-D117N on the function of *Na_v1.5* were suggested to uncover mechanisms leading to conduction disturbances in *zaspopathy*.⁹

Here we present compelling evidence that although *ZASP1*-p.(D117N) is rather prevalent in the Bedouin population of the Negev in Israel, it does not segregate as a causative mutation of DCM,

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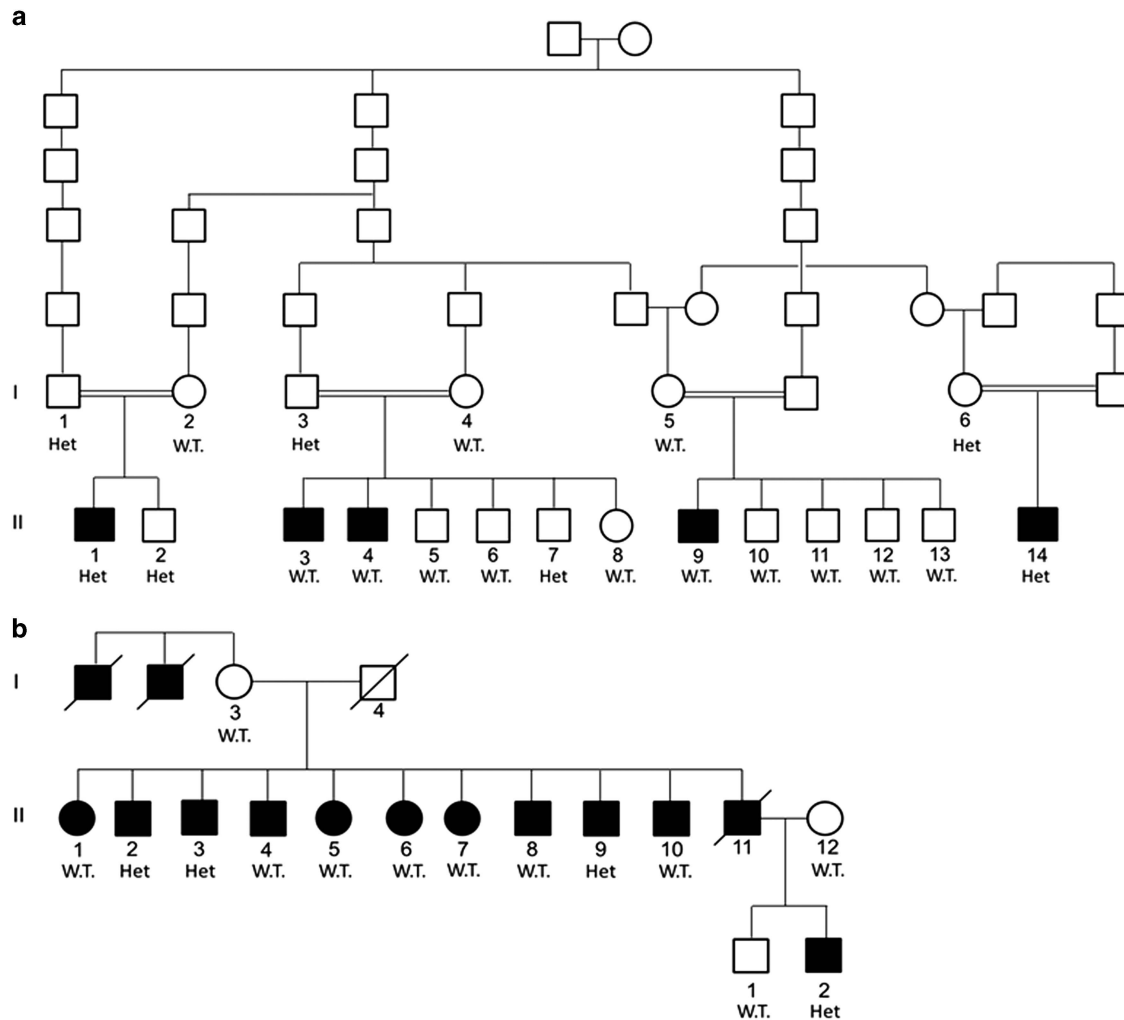


Figure 1 Pedigrees of the families. (a) Family with DCM, focal INLVM, and sustained VT. (b) Family with non-ischemic DCM in young adulthood and a family history of sudden cardiac death and ventricular arrhythmias.

conduction abnormalities or arrhythmic phenotype in our families of interest. Our results indicate that the linkage between ZASP1-p. (D117N) and cardiac pathologies should be further clarified.

SUBJECTS AND METHODS

Patients

The study was approved by the Soroka Medical Center Review Board and the subjects gave informed consent. The patients' medical records were carefully reviewed, and details of their somatic growth, psychomotor development, clinical course, hospitalizations, and laboratory results were recorded. Their parents and siblings were interviewed and underwent a complete physical examination (particularly focused on the cardiac and neuromuscular systems). The patients' evaluation included: echocardiology: transthoracic two-dimensional and Doppler echocardiography performed using a System Vivid 7 echocardiograph (GE Medical Systems, Hatfield, UK). Measurements of left ventricular (LV), LV end-diastolic dimension (LVED), and LV end-systolic dimension (LVES) were obtained in accordance with the recommendations of the American Society of Echocardiography.¹⁰ Fractional shortening (FS) was calculated as $((LVED-LVES)/LVED) \times 100$. Dimensions were corrected for age and body surface area (BSA) according to the formula of Henry *et al*¹¹ ($LVED$ (percent predicted) = (measured LVED/predicted LVED) $\times 100$; predicted LVED = $(45.3 \times \text{body surface area}^{0.3}) - (0.03 \times \text{Age}) - 7.2$). LV abnormalities were classified as follows: DCM, $LVED \geq 117\%$ predicted, and $FS < 25\%$ in the absence of known causes of ventricular dilatation.^{11,12} LV noncompaction

(INLVM) diagnosis was determined according to the echocardiographic criteria described by Jenni *et al*.^{13,14}

DNA preparation and next-generation sequencing and analysis

Genomic DNA was extracted from peripheral blood and submitted to Otogenetics Corporation (Norcross, GA, USA) for exome capture and sequencing. Data were analyzed for data quality, exome coverage, and exome-wide SNP/InDel using the platform provided by DNAnexus (DNAnexus, Inc, Mountain View, CA, USA).

Basic bioinformatics analysis was performed at DNAnexus and Omicia Opal (Oakland, CA, USA; <http://www.omicia.com/>) was used to search for cardiomyopathy known mutations.

Verification of the variant

To verify the variant by Sanger sequencing, PCR amplification of exon 6 was performed using primers F: 5'-ACCCACCATCTGGAGACTTG-3'.

R: 5'-GTCCAACGTGAGGGAAGAAA-3'. PCR conditions were 94 °C for 3 min followed by 35 cycles of 94 °C for 40 s, 62 °C for 40 s and 72 °C for 1 min, respectively, with final extension step of 7 min at 72 °C. Direct sequencing the PCR products was performed on an ABIPRISM3100DNA Analyzer with the BigDye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) according to the manufacturer's protocol.

The data on the population screening was submitted to the LVOD database, individual ID 00046334.

Table 1 Clinical evaluation of family A

| Patient | Age at onset (years) | Presenting symptoms at onset | Age at follow-up (years) and present situation (p/s) | Echo at onset | Echo at follow-up | Cardiac MRI Cine-SSFP | | MRI description Cine-SSFP 4 chambers short axis | LGE |
|---------|----------------------|--|--|---|---|--|--|---|-----|
| | | | | | | LVEDV/ml/sq | LVESV/ml/sq | | |
| I11 | 2 months | Mild congestive heart failure, dyspnea and tachypnea | 5 | LVEDD 22 mm Z-score 5.25 LVEDD 28 mm Z-score 3.28 EF-44% LV dilatation | LVEDD 35 mm Z-score 4.97 LVEDD 44 mm Z-score 2.65 EF-46% LV non compaction | | | | |
| I13 | 16 | Ventricular tachycardia | 18 p/s heart transplantation, presently 22Y | LVEDD 59 mm LVEDD 68 mm EF-35% | LVEDD 60 mm LVEDD 73 mm EF-35% | Severe dilatation LV Severe global LV dysfunction LVEDV-181 LVESV-122 EF-30% | Non compaction on lateral anterior and posterior segments Compact-3.4 mm Non-compact-10 mm | Big patches diffuse/transmural and longitudinal striae of mid-wall enhancement | |
| I14 | 13 | Asymptomatic. Familial cardiomyopathy checkup | 18 p/s ICD at 17.5Y due to VPBS asymptomatic | LVEDD 34 mm LVEDD 51 mm EF-58% | LVEDD 57 mm LVEDD 70 mm EF-35% | Severe dilatation LV Severe global LV dysfunction LVEDV-176 LVESV-114 EF-35% | Non compaction on lateral anterior and posterior segments and apex Compact-6.9 mm Non-compact-1.4 mm | Big patches transmural special apex and mid anterior lateral segments and longitudinal striae of mid-wall of septum enhancement | |
| I19 | 16 | Exercise induced ventricular tachycardia | 21 p/s ICD at 17.5Y due to VPBS. Died at 22 while awaiting transplantation | LVEDD 38 mm LVEDD 54 mm EF = 56% | LVEDD 59 mm LVEDD 65 mm EF < 11% | Not done | | | |
| I114 | 7 | Asymptomatic. Familial cardiomyopathy checkup | 14 p/s asymptomatic, fully active | LVEDD 24 mm LVEDD 43 mm EF-70% | LVEDD 32 mm LVEDD 59 mm EF-70% | Moderate dilatation LV- Normal global LV function LVEDV 91 mm LVESV 39 mm EF-65% | Non compaction lateral anterior and posterior segments Compact-6.4 mm Non-compact-16 mm | No enhancement | |

Abbreviations: EF, ejection fraction; FS, fractional shortening; LV, left ventricular; LVEDD, left ventricular end diastolic diameter; MRI, Magnetic resonance imaging. The annotation of the patients is according to Figure 1a.

RESULTS AND DISCUSSION

Clinical findings

Family A – following several independent hospitalizations of pediatric patients from a single large Bedouin family (Figure 1a), we identified a total of five patients at ages 2 months–18 years who were diagnosed with DCM, focal INLVM, and sustained ventricular tachycardia (VT). Two additional family members who passed away before the study (siblings of patients II-3 and II-4, Figure 1a) were reported to have died from sudden death. Two-dimensional echocardiography in the apical four chamber and parasternal short axis images at the level of the ventricles showed dilatation of both ventricles, with global severe depression of the LV function, 4 and 5 trabeculae and intertrabecular recesses in inferior and lateral walls of the left ventricle with normal origins of the coronary arteries. In these lacunar regions, the ratio of compacted *versus* non-compacted myocardium was 1:2. Cardiac MRI revealed, in short axis, axial and four-chamber images severe dilatation of the left ventricle with severely depressed function. Ejection fraction was calculated to be around 30–65%, with evidence of focal multiple recesses in LV lateral wall. The two older patients were frequently suffering from ventricular tachycardia and on multiple occasions underwent recurrent electrical cardioversion and intravenous administration of amiodarone. They have undergone implantable cardioverter-defibrillator (ICD) implantation and one patient had a heart transplant. The patients' cardiac manifestations are presented in Table 1.

Family B – in the adult cardiac clinic of our institute we identified a large family of Bedouin origin in which an unusually high incidence of non-ischemic DCM in young adulthood and a family history of sudden cardiac death and ventricular arrhythmias are present (Figure 1b). Acquired cardiomyopathy was further ruled out based on past medical history, physical examination, and laboratory tests. Echocardiographic signs of non-compaction were also noted in several siblings. The cardiac manifestations of the family members are depicted in Table 2.

The malignant nature of the disease in this family started after subject II 4 first presented to our cardiology clinic 8 years ago with unexplained syncope, heart failure New York Heart Association class II–III symptoms and non-sustained VT. History revealed that one of the siblings (II 11) died suddenly at the age of 29. Echocardiography revealed severe LV dysfunction, atrial septal defect, and apical trabeculations consistent with a variant of non-compaction syndrome. Coronary angiography revealed normal coronary anatomy and an ICD was implanted for primary prevention of sudden cardiac death. Given his young age and a family history of sudden cardiac death, all other siblings underwent cardiac evaluation for cardiomyopathy, revealing an extremely high incidence of non-ischemic cardiomyopathy in both males and females at the ages of 20–40 years, as depicted in Table 2. Two brothers were diagnosed with moderate to severe LV dysfunction at the ages of 40 and 43 years, without symptoms of heart failure (subjects II 2 and II 8). Both underwent ICD implantation for primary prevention of SCD. Their younger brother (II 3) was found to have mild LV dysfunction with apical trabeculations compatible with a mild variant of non-compaction syndrome. Three sisters (II 5, II 6, and II 7) at the ages of 29, 31, and 37 years, were diagnosed with moderate to severe LV dysfunction, and underwent ICD implantation for primary prevention of SCD. One sister had non-sustained ventricular tachycardia that was recorded by the ICD during follow-up. A fourth sister (II 1) presented with symptomatic right ventricular outflow tract tachycardia that required radiofrequency ablation. An ICD was not implanted yet, given mildly reduced LV function and no evidence of heart failure. Finally, a nephew (III 2) presented at the age of 21 years

Table 2 Clinical evaluation of family B

| Patient | Gender | LDB variant | Age at diagnosis | LV dysfunction | LVEDD (mm) | LVEDS (mm) | Arrhythmia | ICD | Other |
|---------|--------|-------------|------------------|------------------------------|------------|------------|------------|-----|---|
| I3 | F | - | NA | NA | NA | NA | NA | No | |
| I4 | M | NA | Deceased (80) | | | | | | |
| II1 | F | - | 28 | Mild, EF = 50% | 48 | 36 | RVOT VT | No | S/P ablation |
| II2 | M | + | 40 | Moderate to severe, EF = 35% | 51 | 43 | No | Yes | Normal coronary CTA |
| II3 | M | + | 23 | Mild, EF = 50% | 44 | 36 | No | No | Apical trabeculations |
| II4 | M | - | 37 | Severe, EF = 20% | 64 | 53 | VT | Yes | Syncope, ASD, apical trabeculations, normal coronary angiography, NYHA II–III |
| II5 | F | - | 29 | Moderate, EF = 40% | 47 | 38 | No | Yes | |
| II6 | F | - | 37 | Severe, EF = 30% | 47 | 36 | SVT, NSVT | Yes | |
| II7 | F | - | 31 | Moderate to Severe, EF = 35% | 47 | 36 | SVT | Yes | |
| II8 | M | - | 43 | Moderate to Severe, EF = 35% | 55 | 41 | SVT | Yes | Dilated atria |
| II9 | M | + | 37 | Moderate to Severe, EF = 35% | 45 | 40 | RVOT VT | Yes | s/p VT ablation |
| II10 | M | - | 40 | Moderate to Severe, EF = 35% | 47 | 33 | No | Yes | |
| III1 | M | N.A | Deceased (29) | | | | | | |
| III1 | M | - | 26 | NA | NA | NA | NA | No | |
| III2 | M | + | 21 | Severe, EF = 30% | 59 | 38 | NSVT | Yes | |

Abbreviations: ASD, atrial septal defect; CTA, cardiac computerized tomography angiography; ICD, implantable cardioverter defibrillator; LV, left ventricular; LVEDD, left ventricular end diastolic diameter; LVEDS, left ventricular end systolic diameter; NA, not applicable; NSVT, non-sustained ventricular tachycardia; NYHA, New York Heart Association; RVOT, right ventricular outflow tract; SVT, supraventricular tachycardia; VT, ventricular tachycardia; +, presence of variant; -, absence of variant. The annotation of the patients is according to Figure 1b.

Table 3 Clinical evaluation of the healthy individuals carrying the D117N mutation

| Individual | Gender | Age at first examination (years) | Presenting symptoms | Echo at first examination LVEDD | Follow-up (years) | Echo at follow up LVEDD |
|------------|--------|----------------------------------|---------------------|---------------------------------|-------------------|---------------------------------|
| I 1 | M | 16 | Asthmatic | Z-score-1.05 51 mm EF-63% | 12 | Z-score-1.01 51 mm EF-60% |
| I 3 | M | 34 | Asthmatic | Z-score-0.05 47 mm EF-59% | 10 | Z-score-0.055 49 mm EF-59 |
| I 6 | F | 42 | Asthmatic | Z-score-0.56 44 mm EF-68% | 3 | Z-score-0.3 48 mm EF-65% |
| II 2 | M | 6 | Asthmatic | Z-score-0.69 35 mm EF-65% | 3 | Z-score-2 45 mm EF-55% |
| II 7 | M | 9 | Asthmatic | Z-score-0.1 42 mm EF-61% | 5 | Z-score1.1 49 mm EF-62% |

Abbreviations: LVEDD, left ventricular end diastolic diameter; EF, ejection fraction. The numbering of the patients is according to Figure 1a. All ECG measurements were normal.

with severely depressed LV function and non-sustained ventricular tachycardia which necessitated an ICD implantation.

Genetic analysis

The DNA of patients II 1 of family A and II 3 of family B (Figure 1) were exome sequenced. Mutations in genes known to cause cardiomyopathy were verified (100 genes at top of list from genecards (<http://www.genecards.org/>)). No novel variants were found in these genes. The only putative mutation found in heterozygosity in both patients was in Chr10:88446830 (GRCh37) in *ZASP1/ Cypher:LDB3* [NM_001080114.1] c.(349G>A), p.(D117N). This variant was reported to be associated with systolic dysfunction, dilated left ventricle with hypertrabeculated myocardium, and intraventricular conduction disturbance in two white patients,² a 44-year-old female with INLVM and left bundle branch block and a 33-year-old male with INLVM, LV hypertrophy, ventricular bigeminy, and intraventricular conduction delay. We have verified the segregation of this variant in our pedigrees by PCR amplification of exon 6 and sequencing of the PCR products by the Sanger method and found that the variant is not present in all patients and is also present in five healthy members who were thoroughly evaluated, although three of the healthy members could be too young to be informative (Table 3). The segregation of p.(D117N) in *ZASP1/ Cypher* in the families is presented in Figure 1 and Table 2.

Owing to the above findings we have further verified the prevalence of the variant in unrelated individuals from the general Bedouin population from the same region of southern Israel. Eleven out of 210 chromosomes (5.2%) were found to carry p.(D117N) (all in heterozygous state). Although exact epidemiologic data on the prevalence of DCM, ILNCN, idiopathic ventricular arrhythmias, or ventricular conduction abnormalities in this population is lacking, the majority of pediatric and adult cardiac patients are treated by us and we have not observed that sporadic cases of these cardiac presentations are markedly prevalent in the Bedouin population of the region in comparison with other populations or in comparison with numbers in the literature. Thus, the possibility that the p.(D117N) variant is an actual mutation which increases DCM prevalence in the general Bedouin population of the region is highly unlikely. We cannot exclude however, a modifier effect of p.(D117N), that in the genetic background of the Bedouin population has a very low penetrance,

making the p.(D117N) in *ZASP1/ Cypher* less clinically significant in this specific population. However, the fact that we observed no obvious correlation between disease severity and the presence of p.(D117N) in the multiple patients of family B, make this option less likely.

The putative variant designated rs121908338 appears 13 times in the 1000 genomes database (0.65%) and has a prevalence of 1% in the 662 participants of European descent from the ClinSeq project. The Exome Variant Server (<http://evs.gs.washington.edu/EVS/>) presents a prevalence of 0.3% in European Americans and 1.2% in African Americans.

As Bedouins and Arabs are probably under-represented in these variant databases, our study demonstrates that care should be taken when assigning pathogenicity to variants based on low representation in these databases for patients of different populations.

CONCLUSION

Since the segregation pattern in the pedigrees of both families does not show the expected pattern of a causative mutation and this genetic variant occurs in a relatively large proportion of the general population, our results suggest that at least in the Bedouin population (and possibly in other populations) p.(D117N) in *ZASP1/ Cypher* is not the causative mutation for these cardiac abnormalities. This conclusion is further supported by the demonstration that heterozygous patients are not more severely affected than the patients without the p.(D117N) in *ZASP1/ Cypher*. Thus, other causative mutations should be sought.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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